

Redox reactivity of bacterial and mammalian ferritin: Is reductant entry into the ferritin interior a necessary step for iron release?

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ABSTRACT Both mammalian and bacterial ferritin undergo rapid reaction with small-molecule reductants, in the absence of Fe^{2+} chelators, to form ferritins with reduced (Fe^{2+}) mineral cores. Large, low-potential reductants (flavoproteins and ferredoxins) similarly react anaerobically with both ferritin types to quantitatively produce Fe^{2+} in the ferritin cores. The oxidation of Fe^{2+} ferritin by large protein oxidants [cytochrome *c* and Cu(II) proteins] also occurs readily, yielding reduced heme and Cu(I) proteins and ferritins with Fe^{3+} in their cores. These latter oxidants also convert ethetically added Fe^{2+} , bound in mammalian or bacterial apo- or holo-ferritin, to the corresponding Fe^{3+} state in the core of each ferritin type. Because the protein reductants and oxidants are much larger than the channels leading into the mineral core attached to the ferritin interior, we conclude that redox reactions involving the $\text{Fe}^{2+}/\text{Fe}^{3+}$ components of the ferritin core can occur without direct interaction of the redox reagent at the mineral core surface. Our results also suggest that the oxo, hydroxy species of the core, composed essentially of $\text{Fe}(\text{O})\text{OH}$, arise exclusively from solvent deprotonation. The long-distance ferritin-protein electron transfer observed in this study may occur by electron tunneling.

Ferritin is a 24-subunit protein that contains up to 4500 iron atoms in its hollow, nearly spherical interior in the form of a $\text{Fe}(\text{O})\text{OH}$ -type mineral core. The ubiquitous distribution of ferritin among plant and animal species and its presence in nearly every tissue type of highly differentiated organisms have led to the view that ferritin serves as the universal iron-storage protein in nature. In this role, ferritin provides a means for living systems to gain access to this essential mineral nutrient under conditions that otherwise favor the formation of hydrous ferric oxide, a biologically inert form of iron. Having such a control protein that stores iron and regulates the iron flux within its cells allows an organism to maintain effectively the balance between iron insufficiency and iron toxicity. Thus, the delineation of the mechanism by which ferritin functions in its iron-storage and -release roles is important in understanding the entry of iron into various metabolic cellular activities.

Ferritin has been studied extensively (see refs. 1-3 for reviews) with regard to its biochemical characterization as well as its iron-storage and -release function. Of importance in visualizing the overall geometry of the ferritin molecule and details of its subunit arrangements and interactions have been the development and refinement to 2.8 Å of a model (4-6) derived from crystallographic x-ray diffraction studies. Detailed analysis has revealed (6) the presence of channels along the threefold and fourfold axes (3-5 Å across) that penetrate into the central cavity, through which small molecules (Fe^{2+} , reductants, oxidants, and iron chelators) are thought to enter and leave the ferritin interior during the

processes of iron deposition and release (7-14). Iron transport to and from the ferritin interior is a well-established feature of ferritin, but the facile movement of other molecules into and out of the ferritin interior is less well documented. In fact, studies of direct transfer of small molecules into the ferritin interior suggest that moderate diffusional impediments exist with neutral molecules such as sucrose (15, 16) and that serious transfer limitations occur with small anions such as acetate (17, 18), indicating that both charge and size effects are important in channel penetration. Our own recent study (19), using reductants of nearly constant redox potential but of varying charge and cross-sectional area, has shown that the reduction potential is important in effectively reducing the Fe^{3+} core but that the molecular size is quite immaterial. The evidence thus raises questions regarding whether redox agents actually enter into the ferritin interior via the channels as part of the iron-transport mechanism.

These considerations have prompted us to carry out experiments to examine whether penetration of redox reagent into the ferritin interior is a necessary requirement for iron reduction (oxidation) prior to its release (deposition). Here we report that the redox status of the iron in the ferritin core can be altered and that deposition or release can be facilitated by using redox proteins whose dimensions exceed those of the channel openings found within the ferritin molecule. Such results seem to exclude the necessity of reductant or oxidant entry into the ferritin interior as part of the mechanism of ferritin function.

MATERIALS AND METHODS

Mammalian Ferritin. Horse spleen ferritin (2078 Fe^{3+} ions per ferritin molecule) was obtained from Sigma. Ferritin was incubated with 1 mM EDTA or bipyridyl (bpy) to chelate loosely bound metal ions and then passed through a Sephadex G-25 column (1 × 25 cm) to free ferritin of the metal chelates and excess chelator. Three states of native mammalian ferritin were examined: (i) Fe^{3+} -holoferritin, with the core in its native, oxidized Fe^{3+} state; (ii) apoferritin prepared by complete removal of the iron-containing core by the reductive, thioglycolic acid method (20); and (iii) Fe^{2+} -holoferritin, with the core reduced to the Fe^{2+} state but retained within the ferritin interior (21). This latter species was prepared (21, 22) by reduction of holoferritin with excess $\text{Na}_2\text{S}_2\text{O}_4$ and 0.1 mM methyl viologen, a redox mediator, for 30 min under the anaerobic conditions provided by a Vacuum Atmospheres (Hawthorne, CA) glove box (<0.1 ppm O_2 in argon), followed by anaerobic Sephadex G-25 chromatography to remove excess reductant, methyl viologen, and small amounts of Fe^{2+} .

Abbreviations: AvBF, *Azotobacter vinelandii* bacterial ferritin; AvFlp, *A. vinelandii* flavoprotein; AvFlpH₂, reduced AvFlp; AvFdI, *A. vinelandii* ferredoxin I; CtFd, *Clostridium thermoaceticum* ferredoxin; bpy, bipyridyl (in complexes).

Bacterial Ferritin. *Azotobacter vinelandii* (type OP) bacterial ferritin (AvBF) was prepared (23, 24) and was crystallized three times from 0.02 M MgCl₂. The heme content was 12 hemes per 24 subunits (17,300 daltons per subunit) and the core iron content was 1250 iron atoms per AvBF. Four states of AvBF were studied: (i) Fe³⁺-holo-AvBF; (ii) Fe²⁺-holo-AvBF (see procedure for *iii* above); (iii) apo-AvBF with the 12 hemes still attached but with the core iron removed by thioglycolic acid treatment (20); and (iv) Fe³⁺-holo-AvBF with the 12 hemes removed. This latter species was prepared by (a) adjusting an Fe³⁺-holo-AvBF solution to pH 2.0 with 0.2 M HCl, (b) adding methyl ethyl ketone to 50% (vol/vol) to extract the liberated heme groups, (c) adjusting the pH back to 7.0, and (d) centrifuging to remove small amounts of denatured protein.

The purity of mammalian and bacterial apoferritins was verified by sedimentation velocity measurements in a Beckman model E ultracentrifuge. Symmetrical sedimentation patterns were observed for both proteins throughout the sedimentation sequence.

Fe²⁺-Bound Mammalian and Bacterial Ferritin. At pH 8.5 and under strictly anaerobic conditions, mammalian or bacterial holo-ferritin (5–10 mg in 1 ml) was incubated with excess Fe²⁺ (0.01 M FeSO₄·7H₂O in H₂O) for 10 min and passed anaerobically through a Sephadex G-25 column (1 × 26 cm) to remove unbound Fe²⁺. The emerging protein containing bound Fe²⁺ was quantitated by Lowry protein determinations, and the bound Fe²⁺ was determined colorimetrically ($\epsilon = 8400 \text{ M}^{-1}\text{cm}^{-1}$ at 514 nm) by addition of excess bipyridyl. An identical procedure was followed in preparing Fe²⁺-bound mammalian and bacterial apoferritin.

Redox Proteins. *A. vinelandii* flavoprotein (AvFp) and ferredoxin I (AvFdI) were prepared by established procedures (25), as was plastocyanin (26). Stellacyanin (E. Solomon, Stanford University) and *Clostridium thermoaceticum* ferredoxin (CtFd; L. Ljungdahl, University of Georgia) were gifts. Cytochrome *c* was obtained from Sigma. Reduced but S₂O₄²⁻-free AvFdI (AvFpH₂), AvFdI, and CtFd were prepared by reaction with excess S₂O₄²⁻ followed by anaerobic Sephadex G-25 chromatography.

Redox Reactivity. All samples were prepared under the anaerobic conditions provided by a Vacuum Atmospheres glove box equipped with dual purifiers, which maintained an argon atmosphere at <0.1 ppm O₂. Optical samples (1.0 ml, 1.0-cm path length) of Fe²⁺-ferritin (fully reduced or Fe²⁺-bound) were mixed with the oxidized states of the indicated redox proteins and the reaction was monitored optically with a Cary 118 spectrophotometer by the appearance of reduced heme or the disappearance of the 600-nm optical band from the Cu(II) proteins. The disappearance of the $g = 2$ EPR signal of the Cu(II) proteins was also followed at 20 K. Reaction with Fe(CN)₆³⁻ or O₂ was followed by the absorbance change at 400 nm as Fe³⁺ formed within the core or by the decrease in Fe(bpy)₃²⁺ formation upon bipyridyl addition.

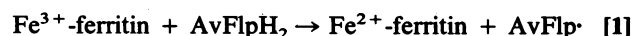
Fully oxidized (but anaerobic) Fe³⁺-ferritin samples were incubated with reduced AvFdI, AvFpH₂, or CtFd and the development of the blue, EPR-active ($g = 2$) semiquinone (AvFpH₂[•]) was monitored optically ($\epsilon = 1980 \text{ M}^{-1}\text{cm}^{-1}$ at 580 nm) or by EPR ($g = 2$) to quantitate electron transfer into the ferritin core (AvFpH₂ → AvFpH₂[•] + e; $E_{1/2} = -515 \text{ mV}$). Because the ferredoxins have only small optical changes between the oxidized and reduced states, reactivity assessment was determined colorimetrically after bipyridyl addition, which liberates Fe(bpy)₃²⁺ only if successful electron transfer from ferredoxin to core iron occurs.

RESULTS

Core Reduction with Proteins. The reaction of excess S₂O₄²⁻ alone or in the presence of methyl viologen (which

facilitates core reduction) completely reduces the Fe³⁺-containing cores of mammalian (21) and bacterial (22) ferritins. Following reduction, anaerobic Sephadex G-25 chromatography provides ferritins from these two species that are free of excess reductant and other small molecules, have intact cores, and are in the Fe²⁺ state. Reaction of these Fe²⁺-ferritins with excess bipyridyl immediately forms the intense red Fe(bpy)₃²⁺ complex, demonstrating the presence of readily removed Fe²⁺ within the core. We have relied extensively, but not exclusively, upon this reaction to demonstrate the presence of Fe²⁺ in the ferritin core after anaerobic reaction of ferritin with large (compared to the channel openings) protein reductants.

Fig. 1 shows a partial optical titration of anaerobic mammalian Fe³⁺-ferritin with AvFpH₂. The most prominent feature is the development of the 570-nm band resulting from the formation of AvFp[•]. The extent of this reaction was also followed by the development of the $g = 2$ EPR spectrum of AvFp[•].



Addition of excess bipyridyl to the optical cell after AvFpH₂ addition immediately forms Fe(bpy)₃²⁺ with the stoichiometry shown above. Because the reduction capacity of the ferritin core is so large (2078 Fe³⁺ ions per molecule), it is not convenient to fully reduce the ferritin core by the difficult-to-prepare AvFpH₂. In experiments of this type, a maximum of 646 of the 2078 Fe³⁺ ions present (31% core reduction) were stoichiometrically reduced. Identical behavior was observed for the reaction of AvBF with AvFpH₂, during which up to 20% of the core Fe³⁺ ions were reduced, with no

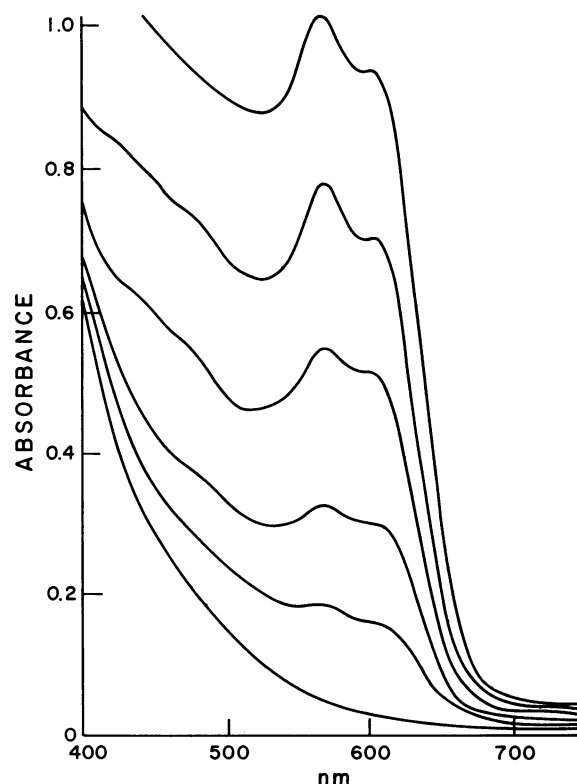


FIG. 1. Reaction of mammalian ferritin with AvFpH₂. Oxidized mammalian ferritin (bottom spectrum) containing 1.41 mmol of Fe³⁺ was incubated sequentially with 40, 40, 55, 55, and 55 μmol of AvFpH₂ (spectra in ascending order). The spectrum of AvFp[•] develops near 570 nm, indicating that the redox couple (AvFpH₂ → AvFp[•] + e) is operative.

reduction occurring with any of the 12 heme groups present. Such results are consistent with a previous report (22) indicating that the heme groups are 30 mV more difficult to reduce than the Fe^{3+} core of AvBF. Although we did not follow the reaction of holo-AvBF with AvFlpH₂ to complete reduction of both the core and the heme groups, we did establish, by the formation of both reduced ferritin heme and AvFId, that the heme groups in apo-AvBF can be reduced by AvFlpH₂.

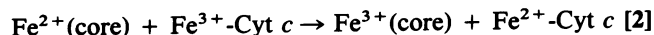
With both types of Fe^{2+} -holoferritin, the formation of $\text{Fe}(\text{bpy})_3^{2+}$ is rapid, occurring within the mixing time of the experiments (<10 sec), and independent of whether AvFlpH₂ is added first and bipyridyl second or whether AvFlpH₂ is added to ferritin solutions already containing bipyridyl.

Both AvBF and horse spleen ferritin react with the two ferredoxins studied (CtFd and AvFId) to form $\text{Fe}(\text{bpy})_3^{2+}$ at rates much slower than that with AvFlpH₂. These reactions are quite complicated, not only because of the absence of convenient optical bands to follow electron transfer by the ferredoxins, but also because some $\text{Fe}(\text{bpy})_3^{2+}$ forms upon reaction of bipyridyl with reduced ferredoxins. By allowing the reactions to proceed for 30 min and by using proper reduced ferredoxin controls, we have shown that more $\text{Fe}(\text{bpy})_3^{2+}$ forms in the presence of the two ferritins (indicating that Fe^{2+} formation occurs in the core) than in their absence. We conclude that these reduced ferredoxins slowly reduce the ferritin cores but are much less effective than AvFlpH₂ or $\text{S}_2\text{O}_4^{2-}$ or other small-molecule reductants.

AvBF with the Fe^{3+} core present but with all heme groups removed undergoes reduction by AvFlpH₂ to form AvFlp- and Fe^{2+} according to reaction 1. In all aspects examined, the heme-free bacterial ferritin behaves identically to mammalian ferritin, which has no hemes in the active state, a result that indicates that the heme groups on AvBF are not essential for Fe^{3+} -core reduction.

Core Oxidation with Proteins. Both mammalian and bacterial ferritin, with reduced Fe^{2+} cores, are conveniently prepared as described and, when exposed to air, are readily oxidized to the respective Fe^{3+} -ferritins, which are spectroscopically (visible/UV and EPR) and analytically indistinguishable from the corresponding native Fe^{3+} -ferritin.

Fig. 2 shows a partial optical titration of mammalian Fe^{2+} -ferritin with anaerobic Fe^{3+} -cytochrome *c*. The rapid development of the reduced heme bands at 550 and 537 nm upon addition of Fe^{3+} -cytochrome *c* clearly establishes that electron transfer occurs from Fe^{2+} -ferritin to cytochrome *c*. Less dramatic and more difficult to measure quantitatively is the overall rise in absorbance between 450 and 300 nm, due to the formation of Fe^{3+} -ferritin, that occurs concomitantly with cytochrome *c* reduction. The reverse titration, which involves the addition of limiting Fe^{2+} -ferritin to excess Fe^{3+} -cytochrome *c*, similarly shows rapid formation of reduced heme and quantitative oxidation of Fe^{2+} to Fe^{3+} in the ferritin core. The two titrations combine to establish the 1:1 stoichiometry shown in reaction 2.



The reaction of Fe^{2+} -ferritin with the two redox-active Cu(II) proteins plastocyanin and stellacyanin was also examined. These copper proteins have strong, distinctive $g = 2$ EPR signals and optical absorption bands near 600 nm ($\epsilon = 4000 \text{ M}^{-1}\text{cm}^{-1}$) in the Cu(II) state that are both absent upon reduction to the Cu(I) state. The reaction of limiting, anaerobic stellacyanin with Fe^{2+} -AvBF or mammalian Fe^{2+} -ferritin quickly eliminates the 600-nm absorbance band and the $g = 2$ EPR signal, characteristic of oxidized stellacyanin, and produces the optically observable Fe^{3+} form of the ferritin species. Plastocyanin undergoes a similar reaction but

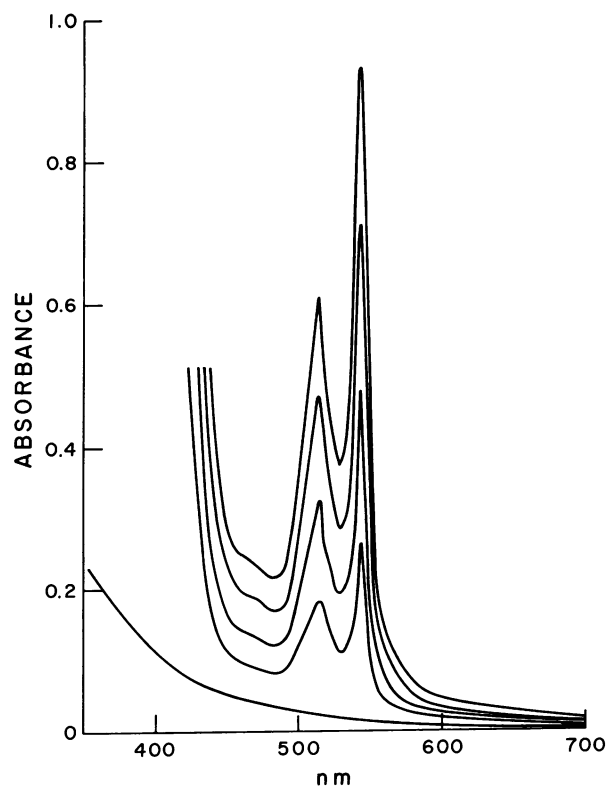


FIG. 2. Reaction of mammalian ferritin with cytochrome *c*. Reduced mammalian ferritin (1 ml) containing 350 nmol of Fe^{2+} (bottom spectrum), determined as $\text{Fe}(\text{bpy})_3^{2+}$, was reacted with 11-nmol aliquots of cytochrome *c* (ascending spectra). The Soret region of the cytochrome *c* spectrum is not shown.

at a much slower rate (20% of the stellacyanin reaction). The reaction stoichiometry by both EPR and optical measurements was determined for reaction of both Cu(II) proteins with both reduced ferritins as shown in reaction 3.



The reaction of mammalian Fe^{2+} -ferritin with the oxidized form of AvFlp and the two ferredoxins studied in the previous section was examined, but no EPR or optical spectroscopic evidence was found to indicate that any reaction occurred. It appears that oxidation of the Fe^{2+} core is not strictly reversible and that reoxidation is only effected by strong protein oxidants, such as heme proteins ($E_{1/2} = 250 \text{ mV}$) and Cu(II) proteins ($E_{1/2} = 350 \text{ mV}$).

Oxidation of Fe^{2+} -Ferritins. The anaerobic addition of excess Fe^{2+} to native mammalian or bacterial Fe^{3+} -ferritin, followed by anaerobic Sephadex G-25 chromatography, conveniently produces Fe^{2+} -bound ferritin that is free of unbound Fe^{2+} ions. We studied, by optical and EPR spectroscopy, the reaction of this ferritin form with cytochrome *c*, plastocyanin, stellacyanin, O_2 , and $\text{Fe}(\text{CN})_6^{3-}$ (this reaction will be discussed below). In all cases, the bound Fe^{2+} was oxidized as evidenced by (i) the production of reduced heme from oxidized cytochrome *c* addition, (ii) the disappearance of the Cu(II) EPR and optical spectroscopic properties of the added copper proteins, and (iii) the absence of $\text{Fe}(\text{bpy})_3^{2+}$ formation upon addition of excess bipyridyl. We have been unable to discern any differences in the reactivity patterns of reduced or partially reduced Fe^{2+} -ferritin and those species produced by Fe^{2+} addition to Fe^{3+} ferritin, indicating that the Fe^{2+} ions in these differently formed ferritins are indistinguishable by the redox agents studied.

The reaction of mammalian and bacterial apoferritin with excess Fe^{2+} followed by anaerobic G-25 chromatography results in the formation of Fe^{2+} -bound apoferritins, free of excess Fe^{2+} . These ferritins undergo reaction with O_2 , $\text{Fe}(\text{CN})_6^{3-}$, and cytochrome *c* to yield the corresponding Fe^{3+} -ferritins with small, but apparently normal, Fe^{3+} cores (Table 1). The reaction with cytochrome *c* is of particular interest because (i) it demonstrates that Fe^{2+} , apparently in the ferritin interior, is readily oxidized to Fe^{3+} by a molecule too large to enter the protein interior and (ii) it shows that the $\text{Fe}(\text{O})\text{OH}$ core is formed without the participation of O_2 or other oxygen-containing oxidants. The oxo and hydroxy groups found in the newly formed $\text{Fe}(\text{O})\text{OH}$ core after cytochrome *c* oxidation of bound Fe^{2+} must have arisen from deprotonation of solvent during the hydrolysis of Fe^{3+} .

Ferricyanide- Fe^{2+} Interaction. Prussian blue is an intensely blue, charge-transfer complex (27) formed by the reaction of free Fe^{2+} with $\text{Fe}(\text{CN})_6^{3-}$. Such an easily detectable reaction product provides a convenient chemical indicator to determine if free or readily detached Fe^{2+} ions are present in any of the Fe^{2+} -ferritin preparations studied here, prepared either by Fe^{3+} core reduction or by Fe^{2+} addition to the ferritin cores. Fe^{2+} , added to a final concentration of 0.01 mM, in 0.15 M NaCl at pH 6.5 rapidly reacts with $\text{Fe}(\text{CN})_6^{3-}$ to form Prussian blue, thereby providing an approximate detectability level of 0.01 mM for Fe^{2+} under the conditions of our experiments. In actual experiments designed to evaluate whether free Fe^{2+} is present in Fe^{2+} -ferritin preparations, the addition of excess $\text{Fe}(\text{CN})_6^{3-}$ to the Fe^{2+} -ferritin solutions (under anaerobic conditions) produced no observable Prussian blue. This result was consistently found for Fe^{2+} -ferritin prepared either by reduction or by Fe^{2+} addition to mammalian and bacterial holo-ferritins, as well as for Fe^{2+} bound to the apoferritins. These results indicate that no free or easily dissociable Fe^{2+} is present to react with $\text{Fe}(\text{CN})_6^{3-}$ to form Prussian blue in the ferritin samples studied. However, upon closer examination, the added Fe^{2+} was found to be Fe^{3+} and strongly attached to the ferritin molecules. Thus, it appears that Fe^{2+} in the presence of ferritin prefers to undergo oxidation to form the ferritin core rather than form Prussian blue, as shown by the reaction pathway to the left in Scheme I. Table 1 summarizes

the results for the reaction of $\text{Fe}(\text{CN})_6^{3-}$ and other oxidants with Fe^{2+} mammalian apoferritin.



Scheme I

As with the cytochrome *c* reaction, $\text{Fe}(\text{CN})_6^{3-}$ oxidizes Fe^{2+} under O_2 -free condition to form $\text{Fe}(\text{O})\text{OH}$ in the ferritin core with the oxo and hydroxy groups presumably coming from solvent deprotonation.

DISCUSSION

The *in vitro* deposition of iron into the ferritin core occurs by the reaction of strong oxygen-containing oxidants (O_2 , IO_3^- , etc.) with Fe^{2+} in the presence of either apo- or holo-ferritin (7–10). One mechanistic proposal (10) for ferritin core formation suggests the actual incorporation of O_2 into the oxo, hydroxy matrix of the core by O_2 diffusing into the ferritin channels and reacting with a channel-bound pair of Fe^{2+} ions. The resulting peroxo-bridged species migrates into the interior, forming Fe^{3+} deposited within the core. Although this proposal and other views (7–10) suggest direct O_2 (or other oxygen-containing oxidants) involvement at or near the developing mineral core, the results reported here show that the oxidant need not participate directly in core development. For example, under anaerobic conditions, $\text{Fe}(\text{CN})_6^{3-}$ is as effective as O_2 in causing core development (see Table 1), even though there are no oxygen atoms present. In the case of O_2 and $\text{Fe}(\text{CN})_6^{3-}$, both molecules are small enough to enter the channels and react with Fe^{2+} in the ferritin interior, thereby initiating (for apo) or propagating (for holo) the ferritin mineral core. However, with cytochrome *c* (Table 1) and the Cu(II) proteins studied here, such channel entry is precluded by the dimensions of the protein oxidants, yet it is clear from the results presented here that indistinguishable core development occurs within ferritin with large or small oxidants or with oxygen-free or oxygen-containing oxidants. Thus, we conclude that oxidant entry into the ferritin interior is not a strict requirement for core oxidation (with subsequent iron deposition). These results suggest that perhaps a previously unsuspected electron-transfer process is operative that causes Fe^{2+} oxidation to occur by an indirect interaction (not at the mineral surface) with external oxidants, outside of the protein interior. After such electron transfer has occurred, the newly formed Fe^{3+} hydrolyzes within the ferritin interior to form the $\text{Fe}(\text{O})\text{OH}$ core with the oxygen atoms arising from the solvent deprotonation.

Recent findings in our laboratory (19) have indicated that a closely related series of small redox molecules all reduce mammalian ferritin at rates more dependent on their redox potentials than on their molecular dimensions. The results reported here for reducing proteins that slowly (AvFdl, CtFd) or rapidly (AvFlpH₂) reduce the iron cores of mammalian and bacterial ferritin demonstrate that reductant entry into the ferritin interior is not a necessary step in core reduction. Thus, the conclusion reached above, that oxidant entry into the ferritin core is not necessary for iron deposition, is reinforced by the reverse reaction of iron reduction and mobilization also not requiring reductant entry into the ferritin interior.

Although the nature of the electron-transfer reaction in either the core-oxidation or core-reduction direction is unclear, we believe our results have eliminated from consideration some possible small-molecule-mediated reactions and, consequently, indicate that a mechanism involving long-range electron transfer is a viable explanation for the observed protein-protein redox reactivity. For example, the reported partial reduction (28, 29) of the ferritin core by

Table 1. Iron content of mammalian ferritin

Oxidant	Content, mol/mol of ferritin					
	Cycle 1		Cycle 2		Cycle 3	
	Fe^{2+}	Fe^{3+}	Fe^{2+}	Fe^{3+}	Fe^{2+}	Fe^{3+}
O_2	52.5	55.3	41.5	116	64.0	185
$\text{Fe}(\text{CN})_6^{3-}$	35.6	39.2	42.2	85.1	41.1	133
Cyt <i>c</i>	28.9	23.5	—	—	—	—

Mammalian apoferritin was incubated first at pH 8.5 with excess Fe^{2+} and passed through an anaerobic Sephadex G-25 column to remove unbound Fe^{2+} . The bound Fe^{2+} content (Fe^{2+} per ferritin) of the emerging protein was then determined by $\text{Fe}(\text{bpy})_3^{2+}$ formation and is listed under Fe^{2+} . The Fe^{2+} -containing protein was then incubated with an excess of the indicated oxidant for 30 min and passed through the G-25 column again to remove excess oxidant [for O_2 and $\text{Fe}(\text{CN})_6^{3-}$], and the Fe^{3+} content (Fe^{3+} per ferritin) was determined and is listed under Fe^{3+} . For cytochrome *c* (Cyt *c*, $M_r \approx 12,000$), separation from ferritin was achieved by anaerobic membrane-selective (nominal molecular weight cut-off = 100,000) concentration-dilution cycles. Headings indicate the number of such successive cycles carried out for each oxidant. Note that for cycles >1, the Fe^{2+} values are for binding to the nascent core, produced in the previous cycle, and the Fe^{3+} values represent the actual Fe^{3+} core content after the indicated cycle. The Fe^{3+} content after oxidation and separation of the newly formed Fe^{3+} -ferritin was sometimes larger than that expected from the Fe^{2+} -ferritin precursor. We attribute this increase to uptake of contaminating iron during the oxidation and separation steps.

Fe^{2+} , itself, could suggest that Fe^{2+} bound in the protein core or channels might serve in some manner as a mediator (28) between external reductants and the ferritin core. Unbound Fe^{2+} is not part of this process, since the methods used to prepare the various Fe^{2+} -containing ferritins were shown, by complete baseline separation between Fe^{2+} -bound ferritin and free Fe^{2+} , to produce Fe^{2+} -ferritins free of unbound Fe^{2+} . The lack of production of Prussian blue upon $\text{Fe}(\text{CN})_6^{3-}$ addition further substantiates the absence of free Fe^{2+} in the various Fe^{2+} -ferritin preparations. Similarly, the possibility that the protein-bound prosthetic groups—FMN, heme, and $\text{Cu}(\text{II})$ —used in this study were the active redox reagents, by dissociating, undergoing redox reaction in the ferritin interior, and then reassociating with their parent protein, seems very unlikely because of the methods of preparation of these auxiliary proteins and because such reactions are inconsistent with the known properties of these AvFlp, heme, and $\text{Cu}(\text{II})$ proteins.

In the case of bacterial ferritin, an earlier suggestion was made (22, 30) that the heme groups that are present might serve as the electron-transfer agent from external reductants to the ferritin core. However, the facile reduction of heme-free, holo-AvBF reported here discredits this view and still leaves unanswered for the bacterial ferritin the same question that is unanswered for the mammalian ferritin: how do electrons reach the core from large external redox reagents?

In addition to reduction mediated by channel or core bound Fe^{2+} , it is possible that long-distance electron transfer occurs by electron tunneling through the 20- to 25-Å ferritin protein shell. This distance is large compared to that between naturally occurring and synthetically added inorganic redox components firmly attached to naturally occurring redox proteins, for which facile intramolecular electron tunneling has been reported (31, 32). This electron exchange occurs over a distance of >15 Å, a distance dictated by the placement of the synthetic redox center relative to the native center, presumably by electron tunneling through the intervening protein matrix. For the facile redox reactions reported here between redox proteins and ferritin, perhaps the proper arrangement of "tunneling-specific" aromatic amino acids in the ferritin protein shell assists and facilitates core redox reactions over this much longer distance. Also, the stability of the Fe^{2+} core and the ease with which biomolecules transfer electrons into and out of the ferritin core continue to make the earlier speculation (30) that ferritin may be an electron-storage molecule, a subject of continuing interest. Such hypotheses are of interest but clearly require further experimentation and careful examination of the ferritin structure.

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